METHOD FOR DIAGNOSIS AND THERAPY OF HODGKIN LYMPHOMAS

The present invention relates to processes for diagnosing and treating Hodgkin's lymphomas (lymphogranulomatosis) based on the expression of the variant exon v10 of the gene CD44 as the molecular target, agents for these processes and the use of these agents.

The highly glycosylated cell surface protein CD44 is involved in the interaction between cells and the extracellular matrix such as migration and activation of leukocytes in inflammation and immune monitoring, precursor formation of leukocytic and myeloid cells in bone marrow and also in the development of lymphoid organs and the interaction of cells with the extracellular matrix (Lesley *et al.*, 1993, Günthert 1993, Pals *et al.*, 1993, Mackay *et al.*, 1994). The human CD44 gene is made up of at least 19 exons, of which at least 12 which code for the extracellular region are alternatively spliced (Screaton *et al.*, 1992). The CD44 gene is transcribed in a number of normal tissues and carcinomas (Fox *et al.*, 1994). Whereas the standard CD44 molecule (CD44s) is ubiquitously found expressed in epithelial and mesenchymal tissues, the various isoforms produced by alternative RNA splicing are found in very limited distribution (Heider *et al.*, 1993). Some of the variant isoforms are involved in the activation of lymphocytes and occur in conjunction with metastasisation (Mackay *et al.*, 1994, Günthert *et al.*, 1991, Rudy *et al.*, 1993, Koopman *et al.*, 1993). Although the expression of variant CD44 has been shown to have a

direct biological role in metastasis formation in carcinoma of the pancreas in rats (Günthert *et al.*, 1991, Seiter *et al.*, 1993), its role in human tumours is as yet unknown.

Various reports have been published showing that certain alternatively spliced forms of CD44 were expressed in human metastatic tumours (Heider *et al.*, 1993 and 1996, Fox *et al.*, 1994, Friedrichs *et al.*, 1995, Kaufmann *et al.*, 1995, Salles *et al.*, 1993, Stauder *et al.*, 1995, Koopman *et al.*, 1993, Tanabe *et al.*, 1993). Studies of the expression of CD44 in non-Hodgkin's lymphomas (NHL) concentrated on analysing the so-called lymphocyte homing receptor CD44H or CD44s (Horst *et al.*, 1990a, Horst *et al.*, 1990b, Jalkanen *et al.*, 1991, Möller *et al.*, 1992). Whereas some authors (Horst *et al.*, 1990a, Jalkanen *et al.*, 1991, Picker *et al.*, 1988, Pals *et al.*, 1989, Fujiwara *et al.*, 1993) found a correlation between increased CD44s expression and unfavourable prognosis, other authors (Terpe *et al.*, 1994) could not confirm these findings. Recently, upregulation of CD44v3 and CD44v6 isoforms was found in NHL with unfavourable pathological status (Koopman *et al.*, 1993, Terpe *et al.*, 1994, Salles *et al.*, 1993, Stauder *et al.*, 1995), whilst variant specific CD44-mAbs were used (Mackay *et al.*, 1994, Koopman *et al.*, 1993, Fox *et al.*, 1993).

Various approaches have been developed for making use of the differential expression of variant exons of the CD44 gene in tumours and normal tissues for diagnostic and therapeutic purposes (WO 94/02633, WO 94/12631, WO 95/00658, WO 95/00851, EP 0531300).

The aim of the present invention was to develop new methods of diagnosing and treating Hodgkin's lymphomas

(lymphogranulomatosis) and preparing agents for such processes.

This aim is achieved by means of the present invention. It relates to processes for diagnosing and treating Hodgkin's lymphomas (lymphogranulomatosis) which are based on the expression of the variant exon v10 of the CD44 gene as a molecular marker or target. Antibody molecules of corresponding specificity are particularly suitable as vehicles for selectively reaching Hodgkin's lymphomas *in vivo*.

Preferred processes are characterised in that an antibody molecule is used which binds specifically to the amino acid sequence SEQ ID NO. 2 (see Sequence Listing).

Other aspects of the present invention are the use of antibody molecules of this kind in the processes according to the invention and agents for performing these processes.

The invention further relates to the use of an antibody molecule which is specific to an epitope within the amino acid sequence which is coded by the variable exon v10 of the CD44 gene, for preparing a pharmaceutical composition for the diagnosis and/or treatment of tumoral diseases. The tumoral disease in question is preferably Hodgkin's lymphoma (lymphogranulomatosis).

The invention further relates to an antibody molecule which is specific to an epitope within the amino acid sequence which is coded by the variable exon v10 of the CD44 gene for pharmaceutical use. Preferably, an antibody molecule of this kind is characterised in that it binds to SEQ ID NO. 2. It may be, in particular, a monoclonal antibody, an Fab- or $F(ab')_2$-fragment of an

immunoglobulin, a recombinantly produced antibody, a recombinantly produced chimeric or humanised antibody or single chain antibody (scFv). Preferably, an antibody molecule of this kind is linked to a radioactive isotope, a radioactive compound, an enzyme, a toxin, a cytostatic, a prodrug, a cytokine or some other immunomodulatory polypeptide.

The nucleic and amino acid sequence of the variant exon v10 of the CD44 gene is known (Screaton *et al.*, 1992, Tölg *et al.*, 1993). These sequences are shown in the Sequence Listing (SEQ ID NO. 1 and 2). The existence of degenerate or allelic variants is unimportant to the performance of the invention; such variants are therefore expressly included.

The invention may be carried out with polyclonal or monoclonal antibodies specific to an epitope which is coded by the exon v10. The preparation of antibodies to known amino acid sequences can be carried out using methods known *per se* (Catty, 1989). For example, a peptide of this sequence may be prepared synthetically and used as an antigen in an immunisation procedure. Another method is to prepare a fusion protein which contains the desired amino acid sequence, by integrating a nucleic acid (which may be prepared synthetically or, for example, by polymerase chain reaction (PCR) from a suitable probe) which codes for this sequence, into an expression vector and expressing the fusion protein a host organism. The fusion protein, optionally purified, can then be used as an antigen in an immunisation procedure and insert-specific antibodies or, in the case of monoclonal antibodies, hybridomas which express insert-specific antibodies, are selected by suitable methods. Such methods are known in the art. Heider *et al.* (1993, 1996)

and Koopman *et al.* (1993) describe the preparation of antibodies against variant epitopes of CD44.

However, for the process according to the invention, it is also possible to use antibody molecules derived from poly- or monoclonal antibodies, e.g. Fab- or $F(ab')_2$-fragments of immunoglobulins, recombinantly produced single chain antibodies (scFv), chimeric or humanised antibodies and other molecules which bind specifically to epitopes coded by exon v10. From a complete immunoglobulin it is possible for example to produce Fab- or $F(ab')_2$-fragments or other fragments (Kreitman *et al.*, 1993). The skilled person is also capable of producing recombinant v10-specific antibody molecules. Corresponding methods are known in the art. Recombinant antibody molecules of this kind may, for example, be humanised antibodies (Shin *et al.*, 1989; Güssow and Seemann, 1991), bispecific antibodies (Weiner *et al.*, 1993; Goodwin, 1989), single chain antibodies (scFv, Johnson and Bird, 1991), complete or fragmentary immunoglobulins (Coloma *et al.*, 1992; Nesbit *et al.*, 1992; Barbas *et al.*, 1992), or antibodies produced by chain shuffling (Winter *et al.*, 1994). Humanised antibodies may be produced for example by CDR grafting (EP 0239400). Framework regions may also be modified (EP 0519596). In order to humanise antibodies, nowadays it is possible to use methods such as PCR (cf. for example EP 0368684; EP 0438310; WO 9207075) or computer modelling (cf. for example WO 9222653). It is also possible to prepare and use fusion proteins such as single chain antibody/toxin fusion proteins (Chaudhary *et al.*, 1990; Friedman *et al.*, 1993). The headings "antibody" and "antibody molecules" should include, in addition to polyclonal and monoclonal antibodies, all the compounds discussed in this section as well as other compounds which are structurally derived

from immunoglobulins and can be prepared by methods known *per se*.

For diagnostic purposes, antibody molecules may be linked, for example, to radioactive isotopes such as $^{131}I$, $^{111}In$, $^{99m}Tc$ or radioactive compounds (Larson *et al.*, 1991; Thomas *et al.*, 1989; Srivastava, 1988), enzymes such as peroxidase or alkaline phosphatase (Catty and Raykundalia, 1989), with fluorescent dyes (Johnson, 1989) or biotin molecules (Guesdon *et al.*, 1979). For therapeutic applications, v10-specific antibody molecules may be linked to radioisotopes such as $^{90}Y$, $^{111}In$, $^{131}I$ or $^{186}Re$ (Quadri *et al.*, 1993; Lenhard *et al.*, 1985, Vriesendorp *et al.*, 1991; Wilbur *et al.*, 1989), toxins (Vitetta *et al.*, 1991; Vitetta and Thorpe, 1991; Kreitman *et al.*, 1993; Theuer *et al.*, 1993) cytostatics (Schrappe *et al.*, 1992), prodrugs (Wang *et al.*, 1992; Senter *et al.*, 1989) or radioactive compounds. The antibody may also be linked to a cytokine or another immunomodulatory polypeptide, e.g. tumour necrosis factor or interleukin-2.

Advantageously, the diagnostic process according to the invention can be used to examine samples from patients, e.g. from biopsies, where there is a suspicion of Hodgkin's lymphoma (lymphogranulomatosis) or where this has already been diagnosed but the tumour requires more accurate characterisation. Variant CD44 molecules which contain an amino acid sequence coded by the variable exon v10 can be detected at the protein level by means of antibodies or at the nucleic acid level by means of specific nucleic acid probes or primers for polymerase chain reaction (PCR). The invention consequently also relates to antibody molecules and nucleic acids which are suitable as probes or primers for such processes, and the use of these antibodies and nucleic acids for the diagnosis and analysis of Hodgkin's lymphomas. For

example, tissue sections can be investigated immunohistochemically with antibodies using methods known *per se*. Extracts or body fluids obtained from tissue samples can also be investigated by other immunological methods using antibodies, e.g. by Western blots, enzyme-linked immunosorbant assays (ELISA, Catty and Raykundalia, 1989), radioimmunoassays (RIA, Catty and Murphy, 1989) or related immunoassays. The samples may be investigated qualitatively, semiquantitatively or quantitatively. The expression of the CD44-splice variant v10 in Hodgkin's disease is associated with aggressive behaviour of the tumour and a high risk of recurrence. This correlates with an advanced stage and poor prognosis of NSHD (nodular sclerosis Hodgkin's disease).

As well as *in vitro* diagnosis, antibody molecules with specificity according to the invention are also suitable for *in vivo* diagnosis of Hodgkin's lymphomas. If the antibody molecule carries a detectable label, the label can be detected for diagnostic purposes, e.g. imaging the tumour *in vivo* or for radioguided surgery, for example. For using antibodies conjugated with radioactive isotopes for immunoscintigraphy (imaging), for example, there are a number of procedures on the basis of which the skilled person can perform the invention (Siccardi *et al.*, 1989; Keenan *et al.*, 1987; Perkins and Pimm, 1992; Colcher *et al.*, 1987; Thompson *et al.*, 1984).

Data obtained by detecting and/or quantifying the expression of the variant CD44 epitope v10 can thus be used for diagnosis and prognosis. It may be advantageous to combine such data with other prognostic parameters, e.g. with the grade of tumour.

Antibody molecules with the specificity according to the invention and optionally linked with a cytotoxic agent

may advantageously be used to treat Hodgkin's lymphomas (lymphogranulomatosis). They may be administered systemically or topically, e.g. by intravenous route (as a bolus or continuous infusion), or by intraperitoneal, intramuscular or subcutaneous injection/infusion. Methods of administering conjugated or non-conjugated antibodies, e.g. complete immunoglobulins, fragments, recombinant humanised molecules etc.) are known in the art (Mulshine *et al.*, 1991; Larson *et al.*, 1991; Vitetta and Thorpe, 1991; Vitetta *et al.*, 1991; Breitz *et al.*, 1992, 1995; Press *et al.*, 1989; Weiner *et al.*, 1989; Chatal *et al.*, 1989; Sears *et al.*, 1982).

The antibody molecules may be formulated in a manner known *per se*. For example, they may be present in aqueous solution, optionally buffered with a physiologically acceptable buffer. A solution of this kind may be characterised by the addition of suitable stabilisers and adjuvants. However, the antibody molecules may also be present in the form of a freeze-dried preparation (lyophilisate) which is reconstituted with a suitable solvent, e.g. water, before use.

In a preferred embodiment of therapeutic application, a humanised v10-specific immunoglobulin or an $F(ab')_2$-fragment thereof is linked with $^{90}Y$ (Quadri *et al.*, 1993; Vriesendorp *et al.*, 1995), $^{131}I$ (Juweid *et al.*, 1995; Press *et al.*, 1995; Thomas *et al.*, in: Catty 1985, p. 230-239), $^{186}Re$ (Breitz *et al.*, 1992, 1995) or another suitable radioisotope and used for radioimmunotherapy of Hodgkin's lymphomas. For example, an antibody molecule of this kind may be linked with $^{90}Y$ using a chelating linker such as ITCB-DTPA (isothiocyanatobenzyl-diethylenetriamine pentacetate), whilst a specific activity of 5-20 mCi/mg, preferably 10 mCi/mg should be achieved. This agent can then be administered to a patient with an antigen-positive

tumour in a dosage of 0.1 to 1 mCi/kg of body weight, preferably 0.3 to 0.5 mCi/kg of body weight, most preferably 0.4 mCi/kg. When the total quantity of protein to be administered is from 2 to 5 mg this may be given in the form of a rapid intravenous bolus injection. In the case of monoclonal antibodies it may be necessary to mix the agent with an excess (e.g. a ten-fold molar excess) of the non-radioactive antibody before administering it; in this case, the preparation is better administered in the form of an intravenous infusion over a period of 15 minutes, for example. The application can be repeated. The treatment can be backed up by bone marrow transplantation.

*Figures*

Fig. 1: A. Individual HRS (Hodgkin and Reed-Sternberg) cells of a patient with no recurrence, reacting with mAb VFF16 (CD44v10). The arrow tips point to non-reactive HRS cells. B. >50% of the HRS cells of patients with a recurrence show reactivity (ABC, x 400).

Fig. 2: CD44v10 expression in HRS cells in various patient groups. It should be noted that patients with a poor clinical progress, i.e. recurrence or bone marrow involvement exclusively show more than 10% positive HRS cells, whereas patients with no recurrence have less 10% positive HRS cells. The difference between these two groups is statistically highly significant.

Fig. 3: RT-PCR analysis using CD44v10-specific primers (right half): the main transcript of about 470 bp in all the probes and a weaker transcript of 660 bp in probes 2, 3 and 4 indicate CD44v10-containing isoforms in all 5 cases. A dominant band of 440 bp when using primers which are specific to the 5'- and 3'-constant region indicates the standard form of CD44 (left half).

## Examples

### Example 1 : Preparation of v10-specific antibodies

The entire variant region of the HPKII type of CD44v (Hofmann *et al.*, 1991) was amplified from human keratinocyte cDNA by polymerase chain reaction (PCR). The two PCR primers *5'*-CAGGCTGGGAGCCAAATGAAGAAAATG-*3'*, positions 25-52, and *5'*-TGATAAGGAACGATTGACATTAGAGTTGGA-*3'*, positions 1013-984 of the LCLC97-variant region as described by Hofmann *et al.* contained an *Eco*RI recognition site which was used to clone the PCR product directly into the vector pGEX-2T (Smith *et al.*, 1988). The resulting construct (pGEX CD44v HPKII, v3-v10) codes for a fusion protein of ~70 kD, consisting of glutathione-S-transferase from *Schistosoma japonicum* and the exons v3-v10 from human CD44 (Heider *et al.*, 1993). The fusion protein was expressed in *E. coli* and then affinity-purified over glutathione-agarose (Smith *et al.*, 1988).

Female Balb/c mice were immunised by intraperitoneal route with the affinity-purified fusion protein according to the following plan:

1st immunisation: 90 µg of fusion protein in complete Freund's adjuvant
2nd and 3rd immunisations: 50 µg of fusion protein in incomplete Freund's adjuvant.

The immunisations were carried out at intervals of 4 weeks. 14 days after the last immunisation, the animals were immunised on three successive days with 10 µg of fusion protein in PBS. The next day, spleen cells from an animal with a high antibody titre were fused with P3.X63-Ag8.653 murine myeloma cells using polyethyleneglycol 4000. The hybridoma cells were then

selected in microtitre plates in HAT medium (Köhler and Milstein, 1975; Kearney *et al.*, 1979).

The antibody titre in the serum and the screening of the hybridoma supernatants were carried out using an ELISA. In this test, first of all, microtitre plates were coated with fusion protein (GST-CD44v3-10) or with glutathione-S-transferase on its own. Then they were incubated with serial dilutions of serum samples or hybridoma supernatants and the specific antibodies were detected with peroxidase-conjugated antibodies against murine immunoglobulin. Hybridomas which reacted only with glutathione-S-transferase were discarded. The remaining antibodies were characterised first in an ELISA with domain-specific fusion proteins (exon v3, exon v5 + v6, exon v6 + v7, exon v8 - v10, exon v10) (Koopman *et al.*, 1993). Their immunohistochemical reactivity was tested on sections of human skin.

Antibodies from the supernatants of the hybridoma clones VFF-14 and VFF-16 bind only to fusion proteins containing a domain which is coded by the exon v10.

**Example 2 : Immunohistochemical examination of tissue samples**

Tissue and patients

37 Paraffin-embedded lymph node samples from 29 patients with NSHD (nodular sclerosis Hodgkin's disease; according to Rye classification) were obtained from the collection of the Pathology Department, University Medical School, Graz, Austria, and divided into three groups; group 1: 11 patients with pretreated NSHD before treatment (5 patients at stage I, 6 at stage II), who had been free

from recurrence for more than 6 years; group 2: 9 patients with pretreated NSHD before treatment (4 at stage I, 5 at stage II) suffered a recurrence of the disease in one to three years. Two to three follow-up lymph node sections of NSHD recurrences from 7 of these 9 patients were also included in this study; group 3: 9 patients with bone marrow involvement at the time of the original diagnosis (stage IV).

Immunohistochemistry

The lymph node samples were stained with the following mAbs: CD44 standard (s) recognised by the mAb SFF2; CD44v5 detected by the mAb VFF8; CD44v6 detected by the mAbs VFF7 and VFF18; CD44v10 detected by the mAbs VFF14 and VFF16. Mab SFF2 recognises an epitope common to all CD44-isoforms. Mabs VFF7 and VFF18 recognise different but overlapping epitopes which are coded by the exon v6. Mab VFF8 is specific to exon v5. Mabs VFF14 and VFF16 react with an epitope which is coded by exon v10.

The immunohistochemistry was carried out on sections treated with microwaves (Gerdes *et al.*, 1992), using the avidin-biotin complex (ABC) peroxidase method (Guesdon *et al.*, 1979). Paraffin sections were dewaxed in xylene, rehydrated and the endogenous peroxidase was blocked with $H_2O_2$ in methanol. The slides were placed in a glass stand and wetted in 500 ml of 0.01 M citrate buffer (2.1 g of citric acid in 1 litre of deionised water, pH adjusted to 6.0 using 2 N NaOH). The microwave treatment was carried out for 35 hours at maximum power (600 W) in a microwave oven (BioRad). After 9 minutes' microwave treatment the evaporated buffer was topped up with deionised water. After the microwave irradiation the solution was cooled for 20 minutes. Then the slides were rinsed in phosphate-

buffered saline (PBS) and immunostained by diaminobenzidine (DAB) development.

For comparison, 10 frozen lymph node samples (3 from patients of group 1, 2 from group 2 and 5 recently collected cases) were also incubated with the mAbs VFF14 (v10) and VFF16 (v10), using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method (Cordell *et al.*, 1984).

For control purposes, sections of normal human epidermis which is known to contain the antigens in question were tested (positive controls). Replacement of the primary antibody by normal serum always produced negative results (negative controls).

As an additional control, immunohistochemical staining for CD44v10 and CD44v6 expression was repeated twice in each case. To confirm the findings, the cases were additionally incubated in a different laboratory using the APAAP method (Cordell *et al.*, 1984).

The percentage of HRS (Hodgkin and Reed-Sternberg) cells stained with the antibodies was graded as 0%, less than 10%, 10-50% and over 50%. Care was taken to ensure that the immunoreactive HRS cells were clearly tumour cells (e.g. by looking for the presence of characteristic nuclear details), particularly in those cases where less than 10% of the HRS cells expressed the antigens in question. All the cases were assessed separately by two of the inventors. The spread of the staining, e.g. on the membrane, in the cytoplasm or both, was recorded, as well as the immunoreactivity in cells which were not HRS cells.

Statistical analysis

CD44 expression patterns were analysed using the Pearson-chi-square calculation and the Mantel-Haenszel test for linear association, by means of the program SPSS for Windows. P-values less than or equal to 0.05 were regarded as significant.

Results of the immunohistochemical staining

Table 1 shows a summary of the results obtained with antibodies directed against CD44s, CD44v5, v6 and v10 in HRS cells. The majority of the antigenic reactivity of the HRS cells was on the cell surface in every case. A variable number of HRS cells yielded cytoplasmic and/or dot-like perinuclear reactivity with or without surface staining, which probably reflected the reactivity of CD44 molecules in the Golgi apparatus or in the endoplasmic reticulum.

*CD44v10 expression correlates with an advanced stage and poor prognosis of NSHD.* CD44s-, CD44v5-(detected by mAb VFF8) and CD44v6-expression (detected by mAb VEF18) in HRS cells was found in the majority of cases, although there was great variation in the number of stained cells (Table 1). CD44v6 expression (detected by mAb VFF7) was found in only a few cases and, when present, was restricted to a minority of HRS cells (Table 1). In patients with no recurrence, CD44v10 expression (detected by the mAbs VFF14 and VFF16) was found in only a very few cases, and the proportion of reactive HRS cells was <10% (Figs. 1A and 2). By contrast, in every case of a patient with a recurrence or initial involvement of the bone marrow, CD44v10 expression was found. In the majority of these cases, there was a clear overexpression of CD44v10 with >50% reactive HRS cells (Figs. 1B and 2). This

overexpression of CD44v10 was also found in the lymph node sections of recurrences studied (Table 2).

Frozen lymph node sections from 3 patients with no recurrence and from 2 patients with a recurrence yielded exactly the same results as paraffin sections. The two antibodies specific to exon v10 (VFF14 and VFF16) showed identical results in the reaction either on the surface and/or in the cytoplasm of the HRS cells.

Using Fisher's exact test, the differences in the CD44 isoform expression between groups of patients with non-aggressive and aggressive NSHD yielded the following p-values: CD44s ($p=0.3625$), CD44v5 ($p=0.2415$), CD44v6 (detected with mAb VFF7) ($p=0.2903$), CD44v6 (detected by mAb VFF18) ($p=0.1836$), CD44v10 (detected by mAbs VFF14 and VFF16) ($p=0.001$). This shows that the different expression patterns of CD44v10 (detected by mAbs VFF14 and VFF16) within these NSHD groups were statistically highly significant. The expression of the CD44 splice variant v10 in Hodgkin's disease is associated with aggressive behaviour of the tumour and a high risk of recurrence. It correlates with an advanced stage and poor prognosis of NSHD.

By contrast with earlier immunohistochemical studies of CD44 expression in conjunction with prognostic relevance in neoplasia of different histogenetic origin, which were carried out exclusively on frozen sections (Koopman *et al.*, 1993, Terpe *et al.*, 1994; Ristamäki *et al.*, 1994, 1995, Stauder *et al.*, 1994, Heider *et al.*, 1993, Horst *et al.*, 1990a,b, Heider *et al.*, 1996, Kaufmann *et al.*, 1995, Mulder *et al.* 1994), the present invention was able to demonstrate that CD44-mAbs can also be used on paraffin-embedded material if microwave treatment is used. This process requires constant fixing and microwave

treatment in order to give reproducible results. In order to validate the immunoreactivity obtained with paraffin-embedded material, the immunohistochemical analysis was carried out in parallel on frozen samples and identical results were obtained. In addition, the results of the CD44v10 expression were confirmed by RT-PCR. For the CD44v6 expression, a correlation could be demonstrated with poor prognosis in NHL (Koopman *et al.*, 1993, Salles *et al.*, 1993, Terpe *et al.*, 1994, Ristamäki *et al.*, 1994, Stauder *et al.*, 1994), breast cancer (Kaufmann *et al.*, 1995) and colon carcinomas (Heider *et al.*, 1993). In the cases of NSHD which we investigated, however, only a few cases were CD44v6-positive and we could not detect any correlation with the prognosis, using two different antibodies against CD44v6. Because these two mAbs used against v6 recognise different epitopes of the exon v6-coded amino acid sequence, this absence of detectable CD44v6 in the majority of cases (see Table 1) cannot be explained by modification or masking of epitopes. By contrast with the frequent expression of CD44v5 in gastric adenocarcinomas (Heider *et al.*, 1993) the data relating to CD44v5 expression within the three groups of NSHD were not statistically significant.

Exon v10, in addition to exons v3 and v6, is a variant exon which is constitutively expressed in lymphocytes (Stauder *et al.*, 1994). Up till now, CD44v10 expression in NHLs has not been systematically analysed and this exon has only rarely been detected in carcinomas (Heider *et al.*, 1996). The present invention demonstrates, by the example of two different antibodies against CD44v10 (VFF14 and VFF16), a statistically significant high regulation of CD44v10 expression in HRS cells of NSHD with a poor prognosis (groups 2 and 3). Both exon v10-specific antibodies showed identical results, both on the surface and also (and/or) in the

cytoplasm of the HRS cells. The detection of CD44v10 expression with two different mAbs is important because, for example, in breast cancer different data were obtained by different authors using different mAbs of the same exon specificity (Friedrichs *et al.*, 1995, Kaufmann *et al.*, 1995). To confirm our surprising results still further, all the cases were independently immunostained in a different laboratory (using a different staining method), with identical results.

The results for the CD44v10 expression in HRS cells of NSHD are the first data which show a correlation between CD44v10 expression and the stage and prognosis of the disease. The methods according to the invention thus provide the doctor with valuable diagnostic and prognostic information on Hodgkin's lymphoma. Moreover, CD44v10 is a suitable molecular target for therapeutic interventions in this disease.

Table 1 : Reactivity of HRS cells

| Patients | % | CD44s SFF2 | v5 VFF8 | v6 VFF7 | v6 VFF18 | v10 VFF14 | v10 VFF16 |
|---|---|---|---|---|---|---|---|
| Group 1 | | | | | | | |
| n = 11 | | n % | n % | n % | n % | n % | n % |
| | > 50 | 3 27.3 | 0 0 | 0 0 | 1 9.1 | 0 0 | 0 0 |
| | 10-50 | 3 27.3 | 1 9.0 | 0 0 | 0 0 | 0 0 | 0 0 |
| | < 10 | 3 27.3 | 6 54.5 | 2 18.2 | 8 72.7 | 4 36.4 | 4 36.4 |
| | 0 | 2 18.1 | 4 36.5 | 9 81.8 | 2 18.2 | 7 63.6 | 7 63.6 |
| Group 2 | | | | | | | |
| n = 9 | | n % | n % | n % | n % | n % | n % |
| | > 50 | 2 22.2 | 1 11.1 | 0 0 | 1 11.0 | 4 44.4 | 4 44.4 |
| | 10-50 | 5 55.6 | 1 11.1 | 1 11.1 | 4 44.5 | 5 55.6 | 5 55.6 |
| | < 10 | 2 22.2 | 7 77.8 | 3 33.3 | 4 44.5 | 0 0 | 0 0 |
| | 0 | 0 0 | 0 0 | 5 55.6 | 0 0 | 0 0 | 0 0 |
| Group 3 | | | | | | | |
| n = 9 | | n % | n % | n % | n % | n % | n % |
| | > 50 | 0 0 | 0 0 | 0 0 | 0 0 | 5 55.6 | 5 55.6 |
| | 10-50 | 6 66.7 | 1 11.1 | 1 11.1 | 2 22.2 | 4 44.4 | 4 44.4 |
| | < 10 | 3 33.3 | 8 88.9 | 0 0 | 7 77.8 | 0 0 | 0 0 |
| | 0 | 0 0 | 0 0 | 8 88.9 | 0 0 | 0 0 | 0 0 |

Table 2: Reactivity (%) of CD44v10 (VFF14 and VFF16) and CD44v6 (VFF18) in HRS cells of patients of group 2 (recurrence)

| Patients | CD44v10 (VFF14) | CD44v10 (VFF16) | CD44v6 (VFF18) |
|---|---|---|---|
| 1 | >50 | >50 | 10 - 50 |
| 1 recurrence | 10 - 50 | 10 - 50 | 10 - 50 |
| 1 recurrence | 10 - 50 | 10 - 50 | 10 - 50 |
| 2 | >50 | >50 | 10 - 50 |
| 2 recurrence | 10 - 50 | 10 - 50 | 10 - 50 |
| 3 | 10 - 50 | 10 - 50 | <10 |
| 3 recurrence | 10 - 50 | 10 - 50 | <10 |
| 4 | 10 - 50 | 10 - 50 | <10 |
| 4 recurrence | 10 - 50 | 10 - 50 | >50 |
| 5 | 10 - 50 | 10 - 50 | 10 - 50 |
| 5 recurrence | 10 - 50 | 10 - 50 | 10 - 50 |
| 6 | >50 | >50 | <10 |
| 6 recurrence | >50 | >50 | 10 - 50 |
| 7 | >50 | >50 | <10 |
| 7 recurrence | 10 - 50 | 10 - 50 | <10 |
| 8 | 10 - 50 | 10 - 50 | 10 - 50 |
| 9 | 10 - 50 | 10 - 50 | >50 |

**Example 3: Use of v10-specific RT-PCR for diagnostic purposes**

Reverse transcription polymerase chain reaction (PCR)

Five cases enabled mRNA to be isolated and were additionally analysed by reverse transcriptase polymerase chain reaction (RT-PCR).

1 µg of total RNA was isolated and reverse transcribed as described in the literature (Günthert *et al.*, 1991). 5 µl of first strand cDNA were amplified with Taq polymerase (Promega, Madison, USA) in a volume of 50 µl, using the buffer conditions recommended by the manufacturer. The concentration of primer was 0.2 mM. In order to test the quality and frequency of cDNA synthesis, a GAPDH-PCR was carried out with oligonucleotides which were homologous to positions 8 - 29 and 362 - 339 of the published GAPDH-cDNA sequence (Allen *et al.*, 1987). Pre-incubation for 5 minutes at 95°C was followed by 25 amplification cycles (30 seconds at 95°C, 1.5 minutes at 62°C) and an extension cycle of 7 minutes at 72°C. Then 10 µl of the reaction were analysed on a 2% agarose gel and the amplification product was inspected under UV light after the gel had been stained with ethidium bromide. In order to amplify cDNAs containing CD44v10, primers were used which were homologous with the 3'-end of exon v10 (positions 986-1013, Hofmann *et al.*, 1991) and with the 5'-constant region of CD44 (positions 513-540, Stamenkovic *et al.*, 1989). In order to amplify isoforms containing CD44 standard, a 3'-constant CD44 primer (positions 934-958, Stamenkovic *et al.*, 1989) was used instead of the CD44v10-specific primer. After 40 amplification cycles (94°C for 30 seconds, 62°C for 1.5 minutes), 10 µl of the reaction mixture were analysed as above. For control purposes, instead of RNA, either distilled water was used

(negative control) or a plasmid containing CD44v3-v10 was used (positive control, Heider *et al.*, 1996).

In the 5 cases in which it was possible to isolate RNA, the RT-PCR analysis confirmed the expression of CD44v10 containing CD44-isoforms (as they were samples which had only recently been obtained, the further progress of the disease in these patients is not yet known). The amplified fragments correspond to CD44 transcripts which contain the constant proportion of CD44 combined with the variant exon v10 (460 bp band) or variant exon v10 plus other variant exons (660 bp band) (Fig. 3, right half). For control purposes, parallel cDNAs were amplified with primers which were specific for the 5'- and 3'-constant region of CD44 (Fig. 3, left hand side), obtaining a prominent band of 440 bp which indicates the standard form of CD44. The molecular-genetic results correlated with the immunohistochemical findings, where in all 5 cases a high proportion of the HRS cells (which represented less than 10% of the total number of cells in a sample) expressed CD44v10 and the majority of the cells (HRS plus non-tumour cells) reacted with the anti-CD44s antibody.

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# SEQUENCE LISTING

(1) GENERAL INFORMATION:

(1) APPLICANT:

(A) NAME: Boehringer Ingelheim International GmbH

(B) STREET: Rheinstrasse

(C) TOWN: Ingelheim

(E) COUNTRY: Germany

(F) POST CODE: 55216

(G) TELEPHONE: 06132-77-2770

(H) TELEFAX: 06132-77-4377

(ii) TITLE OF INVENTION: Process for diagnosis and therapy of lymphogranulomatosis

(iii) Number of Sequences: 4

(iv) COMPUTER-READABLE FORM:

(A) DATA CARRIER: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: Patentln Release #1.0, Version #1.30 (EPO)

(2) DATA ON SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 204 Base pairs

(B) TYPE: Nucleotide

(C) STRAND FORM: both

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Genome-DNA

(ix) FEATURE

(A) NAME/CODE: exon

(B) POSITION:1. .204

(D) OTHER INFORMATION:/product= "Exon v10 of human CD44"
/note= "GenBank accession no. L05419"

/citation= ([1])

(ix) FEATURE:

(A) NAME/CODE: CDS

(B) POSITION:3. .203

(x) INFORMATION ON PUBLICATION:

(A) AUTHORS: Screaton, GR

Bell, MV

Jackson, DG

Cornelis, FB

Gerth, U

Bell, JI

(B) TITLE: Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 89

(F) PAGES: 12160 12164

(G) DATE: December-1992

(K) SIGNIFICANT GROUPS IN SEQ ID NO: 1: From 1 to 204

(xi) SEQUENCE LISTING: SEQ ID NO: 1:

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Gly Ser Thr Thr Leu Leu Glu Gly Tyr Thr Ser His Tyr Pro His Thr
                 20                  25                  30

AAG GAA AGC AGG ACC TTC ATC CCA GTG ACC TCA GCT AAG ACT GGG TCC 143
Lys Glu Ser Arg Thr Phe Ile Pro Val Thr Ser Ala Lys Thr Gly Ser
            35                  40                  45

TTT GGA GTT ACT GCA GTT ACT GTT GGA GAT TCC AAC TCT AAT GTC AAT 191
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(2) DATA ON SEQ ID NO: 2:

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(B) TYPE: amino-acid

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Protein

(xi) SEQUENCE LISTING: SEQ ID NO: 2:

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Glu Ser Arg Thr Phe Ile Pro Val Thr Ser Ala Lys Thr Gly Ser Phe
         35                  40                  45

Gly Val Thr Ala Val Thr Val Gly Asp Ser Asn Ser Asn Val Asn Arg
     50                  55                  60
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Ser Leu Ser
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(B) TYPE: Nucleotide

(C) STRAND FORM: Single strand

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Other Nucleic acid

(A) DESCRIPTION: /desc = "PCR Primer"

(xi) SEQUENCE LISTING: SEQ ID NO: 3:

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(2) DATA ON SEQ ID NO: 4:

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(B) TYPE: Nucleotide

(C) STRAND FORM: Single strand

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Other Nucleic acid

(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE LISTING: SEQ ID NO: 4:

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